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Effects of Food Concentration and Availability on the Incidence of Cloning in Planktotrophic Larvae of the Sea Star *Pisaster ochraceus*

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Abstract. A decade ago, cloning was first observed in the planktotrophic larvae of sea stars obtained from plankton tows. However, no controlled experimental studies have investigated what factors may regulate this remarkable phenomenon. In the present study we offer the first documentation of cloning in the planktotrophic larvae of *Pisaster ochraceus* from the northern Pacific coast. This species was used as a model system to investigate three factors that may influence the incidence of asexual reproduction (cloning) in planktotrophic sea star larvae. In an initial experiment, larvae were reared under nine combinations of three temperatures and three food (phytoplankton) concentrations. Larvae reared at 12–15°C and fed the highest food concentrations grew larger than the other larvae and produced significantly more clones. In a second experiment, qualitatively different algal diets were fed to larvae reared under the conditions found to be optimal in the initial experiment. Up to 24% of the larvae consuming a mixed phytoplankton diet of *Isochrysis galbana, Chaetoceros calcitrans,* and *Dunaliella tertiolecta* cloned, and significantly more clones were produced by these larvae than by those fed monospecific diets. Our experiments indicate that cloning generally occurs after larvae have attained asymptotic body length and only when food is abundant and of high quality. Since larval mortality is considered to be extremely high for marine invertebrates with planktotrophic larvae, production of clones under optimal conditions of temperature and food may serve to increase larval populations when the environment is most conducive to larval growth.

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Introduction

Asexual reproduction and regeneration of missing body parts are well-known phenomena in adult sea stars (Anderson, 1956; Emson and Wilkie, 1980; Shirai and Walker, 1988; Mladenov *et al.*, 1989; Mladenov and Burke, 1994). The capacity for asexual reproduction (cloning) in sea star larvae was first suggested over 60 years ago from observations of the bipinnaria larvae of *Luidia sarsi* (Tattersall and Sheppard, 1934), but subsequent laboratory experiments indicated that these larvae were incapable of asexual reproduction (Wilson, 1978). Only recently has cloning and regeneration of missing body parts been confirmed in planktotrophic larvae of sea stars (Bosch *et al.*, 1989; Rao *et al.*, 1993; Jaeckle, 1994; Vickery and McClintock, 1998; Kitazawa and Komatsu, 2000).

Cloning in the bipinnaria larvae of the sea star *Luidia* sp. was first documented from plankton samples collected in the Sargasso Sea in which clones were observed to develop from buds that formed on the larval arms (Bosch, 1988; Bosch *et al.*, 1989). Shortly thereafter, two additional reports confirmed the occurrence of cloning in the natural environment in two unidentified species of planktotrophic sea star larvae also obtained from plankton samples (Rao *et al.*, 1993; Jaeckle, 1994). In addition to the budding process described above (Bosch, 1988; Bosch *et al.*, 1989), autotomy of the anterior portions of larvae was also observed in one of these studies (Jaeckle, 1994). Recently, brachiolaria larvae of the sea star *Distolasterias brucei* have been reported to undergo cloning in laboratory cultures (Kitazawa and Komatsu, 2000) in a manner identical to that described in previous reports (budding and autotomy). Moreover, cloning in laboratory cultures of the planktotrophic larvae of the brittle star *Ophiopholis aculeata* from the northern Pacific has been reported (Balser, 1998). We reported regen-

eration in planktotrophic larvae of the sea stars *Luidia foliolata* and *Pisaster ochraceus* (Vickery and McClintock, 1998). In addition, we observed similar regenerative capacity in planktotrophic echinopluteus larvae of the sea urchins *Dendraster excentricus* and *Lytechinus variegatus* (Vickery and McClintock, 1999). Thus, among Echinodermata, regenerative capacity in planktotrophic larvae has been demonstrated in sea stars, brittle stars, and sea urchins.

The existence of cloning and regenerative capacity among echinoderms with planktotrophic modes of reproduction suggests that there may be selective and adaptive advantages associated with such life history traits. These processes presumably operate under a suite of energetic constraints and trade-offs, whereby only larvae exposed to the most appropriate conditions would be expected to undergo clonal and regenerative events.

To date, no experimental studies have investigated the factors that regulate cloning in planktotrophic marine invertebrate larvae. Larval development, growth, and survivorship are particularly influenced by temperature and food availability (*e.g.*, George, 1994; Fenaux *et al.*, 1994). These may also be important factors affecting rates of larval cloning (Levitan, 1995; Morgan, 1995). In the present study we offer the first confirmed report of cloning in the planktotrophic larvae of the sea star *P. ochraceus*. We also examined the effects of temperature and both food concentration and availability on growth and cloning in *P. ochraceus* larvae.

Materials and Methods

Larval culturing

Pisaster ochraceus is commonly found in intertidal and shallow subtidal habitats of the U.S. Pacific Northwest. The breeding season of *P. ochraceus* is in the late spring and early summer months in the vicinity of Puget Sound, Washington (Strathmann, 1987). Adult specimens were collected during late spring months in 1997 (for temperature-food experiment) and 1998 (for food availability experiment) from rocky substrates along the shore of East Sound, Orcas Island, and transported to Friday Harbor Laboratories, San Juan Island, Washington. Ovaries and testes were dissected from sexually mature specimens (a single female and a single male). Fertilizable ova were obtained by treating excised ovaries with 1-methyladenine (10^{-4} *M*) (Kanatani, 1969), and sperm were diluted in filtered seawater prior to fertilization. During fertilization, ova were rinsed in filtered seawater to remove excess sperm. Embryos and larvae were reared in 2.5-liter glass jars. The cultures were gently stirred and the seawater was changed every 3 days following the methods outlined by Strathmann (1987). The single-celled algae *Chaetocerous calcitrans, Dunaliella tertiolecta,* and *Isochrysis galbana* were selected for larval diets (Strathmann, 1987). Observations and photographs of larvae were made with both a Wild M-5 dissecting microscope and a Nikon Optiphot-2 compound microscope.

Combined temperature and food-level experiments

Once the bipinnaria larvae developed functional digestive systems they were separated into nine experimental treatments (3 × 3 factorial design) to examine the effects of temperature and food level on rates of cloning. Each experimental treatment combining temperature and food level was replicated three times, and each consisted of about 2400 larvae held in 2.5 l of seawater in a glass jar (approx. 1 larva/ml). Ambient spring seawater temperatures (12–15°C) were bracketed in increments of about 5°C to yield treatment groups of low (7–10°C), medium (12–15°C), and high (17–20°C) temperature. Ambient seawater temperatures along the northern Pacific coast generally fall within the middle temperature range (12–15°C) throughout the year, but it is likely that larvae encounter lower temperatures (7–10°C) at the northern limit of their biogeographic range and in deeper water, or during the late winter months (Cannon, 1978). Similarly, larvae may encounter higher temperatures (17–20°C) at the southern limits of their biogeographic distribution, or in surface waters during the early summer months (Cannon, 1978; Strathmann, 1987).

Three levels of food composed of equal cell numbers (as determined using a hemocytometer) of the phytoplankton species *Chaetocerous calcitrans, Dunaliella tertiolecta,* and *Isochrysis galbana* were proffered to larvae in each treatment. The three concentrations of mixed algal cells were 5×10^2, 5×10^3, and 5×10^4 cells/ml (modified after Basch, 1996; Fenaux *et al.*, 1994). The nine experimental treatments combining temperature and food level were therefore as follows: high temperature and high food (HT-HF), high temperature and medium food (HT-MF), high temperature and low food (HT-LF), medium temperature and high food (MT-HF), medium temperature and medium food (MT-MF), medium temperature and low food (MT-LF), low temperature and high food (LT-HF), low temperature and medium food (LT-MF), and low temperature and low food (LT-LF). Subsamples of larvae in each experimental treatment ($n = 200$) were examined every 3 days under a dissecting microscope. The numbers of clones and of larvae undergoing clonal reproduction as evidenced by budding were recorded. The lengths of the larvae in each subsample of each experimental treatment and of all larvae in the process of cloning were measured along the larval axis (George, 1994). Clones and larvae undergoing cloning were placed in separate containers and monitored every 3 days to determine whether clones successfully developed into normal functional larvae and metamorphosed into juveniles. Larval lengths were compared within temperature and food-level experiments using analysis of variance. Only

probability levels where $P \leq 0.05$ were considered statistically significant.

Food-availability experiment

To examine the effects of food availability (phytoplankton type) on the rate of cloning, simulating conditions in which nutrient diversity might be limited, larvae of *Pisaster ochraceus* were obtained as described above using ova and sperm from a different set of parent sea stars than those used for the combined temperature and food-level experiment. The larvae were cultured at 12–15°C at a density of about 2400 larvae per 2.5-l of seawater. Larvae were fed 5×10^4 cells/ml of either a monospecific diet of *Chaetocerous calcitrans, Dunaliella tertiolecta,* or *Isochrysis galbana* or a mixed diet composed of an equal cell number of these three algae. Each of the four experimental treatments was replicated three times. Subsamples of larvae ($n = 200$) were examined every 3 days under a dissecting microscope and analyzed using the same methods as for the combined temperature and food-level experiment. Larval lengths were compared within food availability experiments using analysis of variance. Only probability levels where $P \leq 0.05$ were considered statistically significant.

Results

Combined temperature and food-level experiments

Bipinnaria larvae exposed to seawater temperatures of 12–15°C and fed the highest food level (MT-HF) attained lengths significantly greater than those of larvae in any other experimental treatment (Fig. 1). Moreover, cloning occurred only in this experimental treatment (Fig. 2). Thirty-four days after fertilization, larval clones were first observed in the MT-HF bipinnaria culture (1.2%, Fig. 2). Subsequently, small numbers of additional larval clones were observed. Once bipinnaria in this treatment had doubled in length while developing into brachiolaria larvae (at about day 45), a fivefold increase was observed in the incidence of cloning (6%, Fig. 2).

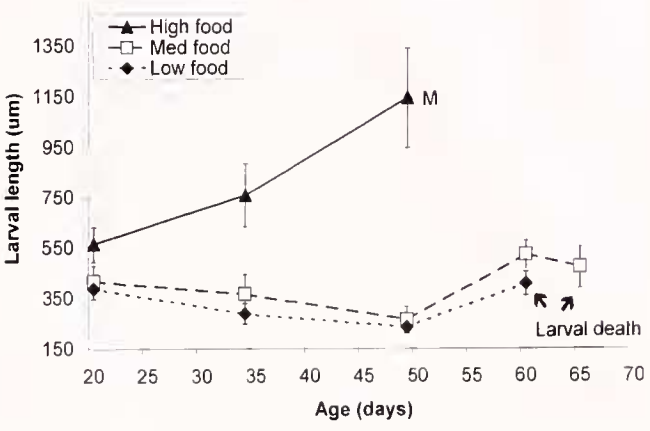

Figure 1. Growth of larvae of *Pisaster ochraceus* (measured as changes in length) reared at 12–15°C and fed low, medium, and high levels of mixed phytoplankton. M indicates stage at which brachiolaria larvae developed an adult rudiment and a decrease in length of the larval body (indicating metamorphic competence). Larvae that reached metamorphic competence were later observed to complete the metamorphosis to juveniles. Error bars represent mean values ±1 SD.

Figure 2. Percentages of larvae of *Pisaster ochraceus* undergoing cloning when reared at 12–15°C and fed high levels of mixed phytoplankton. Diagram indicates approximate onset of brachiolaria stage. Error bars represent mean values ±1 SD.

Larval clones obtained from the MT-HF cultures were isolated and their development was followed through metamorphosis. The clones produced resulted from the regeneration of anterior and posterior portions of bipinnaria and brachiolaria larvae (Fig. 3A, B) by processes that closely resembled those described in detail by Vickery and McClintock (1998). After about 2 weeks, fully developed clonal larvae were functionally and morphologically indistinguishable from larvae in the cultures from which they were originally isolated. A number of bipinnaria and brachiolaria larvae in the MT-HF cultures had missing larval arms or were missing small fragments of the larval body. Some larvae with missing larval arms were in the process of cloning, as evidenced by the development of projections or buds, which later became functional larvae, at the site of the missing fragment (Fig. 3C). However, some larvae with missing larval arms did not form projections or buds, but instead regenerated the larval arm. In addition, some small fragments of larval body parts, including severed larval arms, were observed in the MT-HF cultures, presumably the result of damage incurred when the water in the larval culture was changed. A number of these fragments, including severed arms, were separated from the cultures and observed for 2 weeks. During this time the fragments neither grew nor formed clones, although no mortality was observed.

Those bipinnaria larvae exposed to the highest temperature treatments (HT-LF, HT-MF, and HT-HF) all died

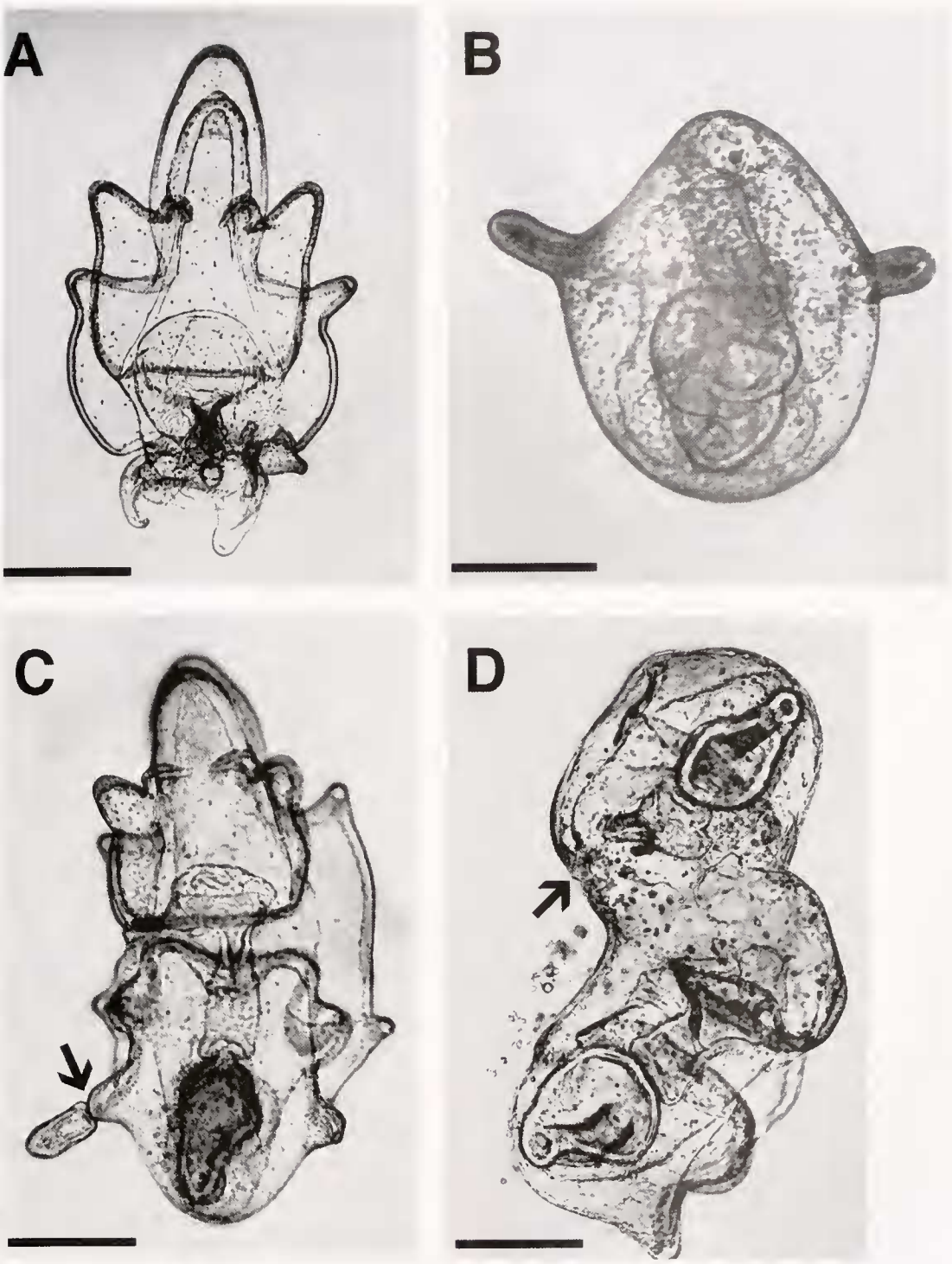

Figure 3. Light photomicrographs of clonal larvae of *Pisaster ochraceus* including the anterior (A) and posterior (B) portion of bipinnaria larvae. (C) Bipinnaria larva in the process of budding (see arrow). The bud later formed an early bipinnaria stage larva and subsequently detached (similar to that described by Bosch *et al.*, 1989). (D) Bipinnaria larva in the process of autotomization by fission. This larva was observed for about 2 weeks, during which the secondary larva (see arrow) detached and became functionally and morphologically indistinguishable from the primary larva in a manner similar to that described by Jaeckle (1994). We have observed this type of autotomy in several other species of planktotrophic larvae (pers. obs., M. Vickery). Scale bars = 200 μm.

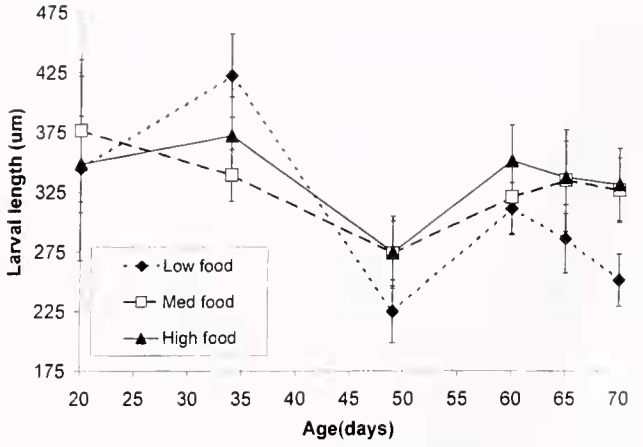

Figure 4. Growth of larvae of *Pisaster ochraceus* (measured as changes in length) reared at 7–10°C on low, medium, and high levels of mixed phytoplankton. Error bars represent mean values ±1 SD.

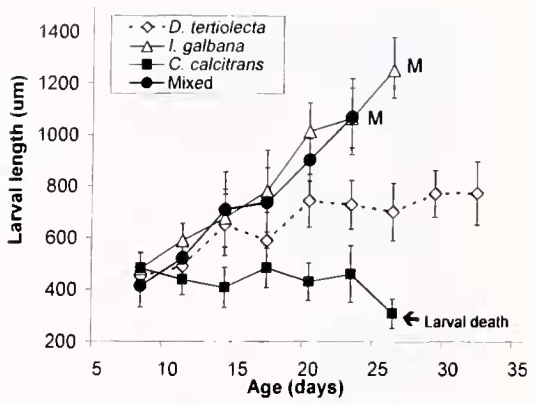

Figure 5. Growth of larvae of *Pisaster ochraceus* (measured as changes in length) reared at 12–15°C and fed high levels of four different phytoplankton diets. M indicates stage at which brachiolaria larvae developed an adult rudiment and a decrease in length of the larval body (indicating metamorphic competence). Larvae that reached metamorphic competence were later observed to complete the metamorphosis to juveniles. Error bars represent mean values ±1 SD.

within one week; no cloning occurred in the short period before mortality. Unlike larvae held at mid-range temperatures, bipinnaria larvae reared at low temperatures (7–10°C) and fed any of the three levels of food (LT-LF, LT-MF, and LT-HF) never developed into brachiolaria larvae over a 70-day period. They remained morphologically identical to early-stage bipinnariae. Moreover, no clones were produced. Larvae presented any food level at low temperature did not increase in length. In fact, they were slightly reduced in length by the end of the 70-day observation period (Fig. 4). Considerable larval mortality occurred throughout the experiment in all low-temperature treatment groups.

Food availability experiment

Bipinnaria larvae in experimental treatments fed a monospecific diet of *I. galbana* or an equivalent density of a mixture of equal cell numbers of *C. calcitrans, D. tertiolecta,* and *I. galbana* grew significantly larger than larvae fed monospecific diets of *C. calcitrans* or *D. tertiolecta* (Fig. 5). The incidence of cloning was greatest (24%) on a mixed diet during and after the transformation from the bipinnaria to the brachiolaria stage (Fig. 6). Although temperature and food concentration were similar in this treatment and in the MT-HF treatment group of the combined temperature and food-level experiment (Figs. 1, 2), growth was more rapid, and cloning began earlier and was more frequent. This might be attributed to the high variability in larval development rate in batches of larvae of *P. ochraceus* (Strathmann, 1978). Also, larvae used for this experiment were the offspring of a different set of parents than those used in the previous experiments.

Discussion

Cloning and regenerative capacity in echinoderm larvae has only recently been documented (Bosch, 1988; Bosch *et al.*, 1989; Rao *et al.*, 1993; Jaeckle, 1994; Balser, 1998; Vickery and McClintock, 1998, 1999; Kitazawa and Komatsu, 2000). In the present study we provide the first documentation of cloning in the planktotrophic larvae of *Pisaster ochraceus.* The results of our bifactorial analysis indicated that both seawater temperature and food level are important factors affecting growth and survival—and therefore cloning—of *P. ochraceus* larvae. Simulated environmental conditions that produced normal development and optimal larval growth generated the greatest number of clones. Larvae reared at temperatures (12–15°C) with the most abundant food exhibited normal development and positive growth, which resulted in the highest survival, with the greatest incidence of cloning. Although we observed rapid larval growth in the high-temperature treatment (15–17°C), none of the larvae survived beyond one week. One possible

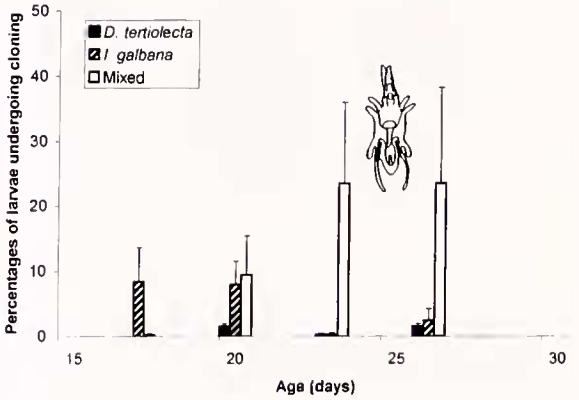

Figure 6. Percentages of larvae of *Pisaster ochraceus* undergoing cloning when reared at 12–15°C and fed high levels of four different phytoplankton diets. Error bars represent mean values ±1 SD.

explanation for the high mortality is that the higher temperatures triggered an increase in bacterial and microalgal growth in the cultures.

In contrast, sea star larvae reared in the low-temperature treatments (7–10°C) showed no net positive growth, and in most cases, decreased in length, regardless of food availability. These larvae also failed to attain the brachiolaria stage of development. It is unlikely these larvae would eventually become clonal because they continued to shrink in length over the course of the experiment. Decreased rates of growth and development at low temperatures may be related to decreased rates of larval metabolism (Boidron-Métairon, 1995). While low seawater temperatures have been suggested as an indirect cause of mortality in marine invertebrate larvae (Thorson, 1950), no studies of larval culturing have shown that low temperature can actually lead to a decrease in larval length as seen in the present study.

The production of larval clones was greatest during phases of rapid larval growth in MT-HF condition. As *P. ochraceus* in the North Pacific spawns in the late spring, larvae typically encounter moderate seawater temperatures (12–15°C) and high phytoplankton availability (Cannon, 1978). Such conditions could be expected to enhance *in situ* rates of larval cloning. Further analysis indicated that presenting larvae with different levels and types of food under an optimal regime of seawater temperature had a pronounced effect on the initiation and rate of larval clone production.

The greatest numbers of clones were produced by larvae in cultures presented a mixture of three single-celled algae. Although monospecific patches of single-celled algae are unlikely to exist in the natural environment, our use of monospecific algal diets simulated conditions in which nutrient diversity might be limited. Thus some of the observed differences in growth (and cloning) rates among the larvae fed monoalgal diets may have resulted from differences in the nutrient content of the food rather than in the type of food, since the larvae were fed equal cell numbers of algae, not an equal nutritional content (Pechenik and Fisher, 1979). However, the amount of nutrients actually consumed by the larvae does not necessarily have any correlation with the nutrient content of the food presented, as some food types may be more palatable to the larvae than others. Future studies may shed more light on this subject. The important information gained from the food-availability experiment is that nutrient availability may be an important factor affecting larval growth and therefore the rate of cloning, as evidenced by the fact that growth rates among larvae fed a monoalgal diet of *Isochrysis galbana* were similar to those fed a diet of mixed algae, yet the larvae fed the mixed diet produced far more clones.

In adult echinoderms, cloning (fission) is common and has been well described (Emson and Wilkie, 1980). Seasonal fluctuations in the incidence of cloning in adult sea stars, especially a high incidence in summer months, have been related to periods of maximum growth (Emson and Wilkie, 1980). This suggests that suitable biotic and environmental conditions such as abundant food and moderate temperatures may trigger cloning processes in adults just as they did in the larvae studied here. In some instances, more than 50% of the adults in a population were observed undergoing cloning (fission) when conditions were optimal (Emson and Wilkie, 1980).

Cloning may serve as a mechanism to enhance recruitment in *P. ochraceus* and perhaps in other marine invertebrates with planktotrophic modes of reproduction. Larvae dispersed across significant distances are likely to encounter a variety of environmental and biotic conditions, and our results suggest that those larvae encountering favorable conditions may be stimulated to reproduce by cloning, thereby possibly increasing the probability of successful larval metamorphosis and juvenile recruitment. Future studies of the effects of larval cloning on larval survivalship and recruitment will provide more insight into the true impact of this phenomenon on the life history of sea stars with planktotrophic larvae.

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